**Boosting proteome depth of coverage by ion mobility fractionation in combination with PASEF approaches on a TIMS-QTOF instrument**

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Ion mobility-based separation coupled to high-resolution mass spectrometry has greatly enhanced our ability to obtain deeper proteome coverage in a single-shot analysis. We report a straightforward approach to obtain comprehensive proteome coverage by gas-phase fractionation utilizing ion mobility separation and DDA-PASEF on the timsTOF Pro. Using HeLa cell lysate as a representative human proteome standard, we identified over 80,000 peptides corresponding to ~8,000 proteins with minimal sample consumption and instrumentation time while still maintaining high quantitation precision. We then explored the utility of this approach to generate spectral libraries for DIA-PASEF analysis of the human proteome in a 20-minute gradient (30-minute instrument runtime). Using the custom, targeted spectral library obtained by ion mobility fractionation, we identified approximately 6,600 and 4,000 proteins on average per run from 200 ng and 10 ng HeLa digest using FragPipe tools and subsequent DIA-NN analysis. Importantly, these custom libraries show comparable coverage to the more comprehensive in silico library analysis, highlighting their general suitability in shorter gradient (<30 min) DIA applications while significantly increasing computational time (~10x) by limiting the library to more experimentally relevant precursors. Further improvement in throughput using this approach can now be obtained by incorporating GPU-enabled, real-time database searching and DIA analysis with PaSER. Results from TIMScore-generated ion mobility fractionated libraries and subsequent TIMS-DIANN analysis in real-time will be discussed.